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PROGRESS IN DESIGN OF BIODEGRADABLE POLYMER-BASED MICROSPHERES FOR PARENTERAL CONTROLLED DELIVERY OF THERAPEUTIC PEPTIDE/PROTEIN

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5.2.1 INTRODUCTION

At the cellular level, deoxyribonucleic and ribonucleic acids (DNA and RNA, respectively) serve as an endogenous vehicle not only to store genetic information but also to transfer genetic information from one generation to their offsprings of all known living organisms. In addition, utilizing the rule of complementary base pairing, the DNA undergoes replication and transcription processes to produce respectively a new double-stranded DNA molecule and a complementary single-stranded RNA molecule. Following the translation process, peptide and protein are synthesized/constructed in ribosomal subunits through peptidic linkages between available 20 amino acids. The peptide and protein thus constructed perform a wide variety of functions and each cell contains several thousands of different proteins. Peptide- and protein-mediated, important physiological and biological processes of the human body include ligands/hormones for signaling, enzymes for biotransformation reactions, receptors for pharmacological response elucidation, antibodies in immune system interactions, transcription, and translation. Hence these molecules play a vital role to ensure proper development and functioning of entire organs of the human body.

Webster's New World Dictionary defines a drug as "any substances used as a medicine or as an ingredient in a medicine." Indeed, peptides and recombinant proteins are highly potent, relatively macromolecular and promising therapeutic agents that emerged out from the significant development of biotechnic and biogenetic engineering technologies. Peptide and protein therapeutics include semisynthetic vaccines, monoclonal antibodies, growth factors, cytokines, soluble receptors, hormones, and enzymes. The advent of recombinant DNA technology allowed the possibility of the commercial production of proteins for pharmaceutical applications from the early 1980s and, in fact, manufacture of therapeutic proteins represented the first true industrial application of this technology [1]. During the 1980s the term *biopharmaceutical* became synonymous with *therapeutic protein produced by recombinant DNA technology* (or, in the case of a small number of therapeutic monoclonal antibodies, *by hybridoma technology*). Clinical evaluation of nucleic acid-based drugs used for the purposes of gene therapy and antisense technology commenced in the 1990s, and today the term biopharmaceutical also encompasses such (as-yet-experimental) drugs [2]. The first such recombinant therapeutic protein (insulin) was approved for general medical use only 24 years ago. Today there are in excess of 100 such products approved in some world regions at least, with 88 having received approval within the European Union (EU). This represents 36% of all new drug approvals since the introduction of the new centralized European drug approval system in 1995 [3]. Over the coming decade, therefore, in the region of a dozen new therapeutic proteins should, on average, gain regulatory approval each year. While EU figures are difficult to locate, the American Association of Pharmaceutical Researchers and Manufacturers (PhRMA) estimates that there are currently some 371 biotechnology medicines in development [4]. Out of these 371 biotechnology medicines, as estimated by PhRMA, more than 300 are protein based, with recombinant vaccines and monoclonal/engineered antibodies representing the two most promising categories. Incidentally, all 88 biopharmaceutical products currently approved within the EU are protein based. Of the proteins thus far approved, hormones and cytokines represent the largest product categories (23 and 18 products, respectively). Hormones approved include several recombinant insulins, displaying both native and modified amino acid

sequences. In addition, several recombinant gonadotrophins [follicle-stimulating hormone (FSH), luteinizing hormone (LH), and human chorionic gonadotrophins (hCG)] have been approved for the treatment of various forms of subfertility/infertility. Cytokines approved include a range of recombinant hematopoietic factors, including multiple erythropoietin-based products used for the treatment of anemia as well as a colony-stimulating factor aimed at treating neutropenia. Additional approved cytokines include a range of interferon-based products, mainly used to treat cancer and various viral infections, most notably hepatitis B and C, and a recombinant tumor necrosis factor (TNF) used as an adjuvant therapy in the treatment of some soft tissue cancers. Blood-related approved therapeutic proteins include a range of recombinant blood coagulation factors used to treat hemophilia, recombinant thrombolytics, and recombinant anticoagulants. Additional product categories include a range of subunit vaccines containing at least one recombinant component [mainly hepatitis B surface antigens (HBsAg)] and a variety of monoclonal antibody-based products indicated for the treatment/detection of various cancers or the prevention of organ transplant rejection. In summary, ailments that can be treated more effectively by this new class of therapeutic agents include cancers, autoimmune disease, memory impairment, mental disorders, hypertension, and certain cardiovascular and metabolic diseases [5, 6].

Poor absorption and easy degradation by endogenous proteolytic enzymes present in eye tissues, nasal mucosa, and gastro intestinal tract and low transdermal bioavailabilities due to relatively large size make the peptide/protein molecules to be administered only through parenteral routes either by multiple injections or infusion therapy in order to achieve desired therapeutic plasma levels for prolonged periods of time. Nevertheless, because of remarkably short half-lives within the *in vivo* arena, the therapeutic usage of most of the peptide/protein is practically possible only through daily multiple injections under close medical supervision. Hence, the commercial success of peptides/proteins as therapeutic agents depends mainly on development of novel drug delivery systems which could potentially reduce the injection frequencies and thus eliminate the accompanying serious problem of patient compliance.

Among the several technologies that have been suggested for reducing injection frequencies of therapeutic peptide/protein, microspheres prepared from biodegradable polymers are widely recognized for controlled drug delivery following parenteral administration. Polyester polymers such as poly(lactic acid) (PLA), poly(glycolic acid) (PGA), and their copolymer poly(lactic acid-*co*-glycolic acid) (PLGA) are used routinely for the preparation of injectable microspheres after taking into consideration their well-known biocompatibility, controlled biodegradability, absorbability, and no toxicity of degradation products [7]. Furthermore, the PLGA types and related poly(hydroxyalkanoates) have a long history of medical and pharmaceutical use in fields as diverse as sutures, bone fixatives, artificial skins and cartilages, dental materials, materials for bone generation, drug delivery, and many others, as reviewed by Ueda and Tabata [8]. In conjunction with a long safety record of PLGA polymers, at least 12 different peptide/protein-loaded PLGA microsphere products are available in the market from nine different companies worldwide for the treatment of some life-threatening diseases (Table 1). In recent years, poly(ϵ -caprolactone) (PCL) has been investigated as an alternative to PLGA to make microspheres [9, 10]. A glimpse of ongoing research activities utilizing biodegradable polymer-based microspheres for various peptide/protein is shown in

TABLE 1 Currently Marketed Preparations (Injectable Microspheres) Containing Peptide/Protein Molecules

Commercial Name	API	Polymer	Company	Indication
Lupron Depot	Leuprolide	PLGA or PLA	TAP	Prostate cancer, endometriosis
Enantone Depot	Leuprolide	PLGA or PLA	Takeda	Prostate cancer, endometriosis
Trenantone	Leuprolide	PLGA or PLA	Takeda	Prostate cancer, endometriosis
Enantone Gyn	Leuprolide	PLGA or PLA	Takeda	Prostate cancer, endometriosis
Sandostatin LAR	Octreotide	PLGA-glucose	Novartis	Acromegaly
Nutropin	Somatropin	PLGA	Genentech	Growth deficiencies
Trelstar Depot	Triptorelin	PLGA	Pfizer	Prostate cancer
Decapeptyl SR	Triptorelin	PLGA or PLA	Ipsen-Beaufour	Prostate cancer
Decapeptyl	Triptorelin	PLGA	Ferring	Prostate cancer
Suprecur MP	Buserelin	PLGA	Aventis	Endometriosis
Somatuline LA	Lanreotide	PLGA	Ipsen-Beaufour	Acromegaly
Parlodel LAR	Bromocriptine	PLGA-Glu	Novartis	Parkinsonism

Abbreviations: PLA: polylactide; PLGA: poly(lactide-co-glycolide); API: active pharmaceutical ingredient; PLGA-Glu: poly(D,L-lactide-co-glycolide-D-glucose).

Table 2 (incorporating refs. 12–35). However, overcoming the propensity for peptides/proteins to undergo degradation processes during incorporation into the biodegradable microspheres or after injection into the body awaiting release is one of the key hurdles in bringing microencapsulated systems for these drugs to market. This partially explains the limited and only a countable number of formulations available on the market. Furthermore, irrespective of the various microencapsulation techniques adopted to prepare peptide/protein-loaded microspheres, several transfer-required processes such as filtration, centrifugation, and vacuum or freeze drying are necessary to obtain a final product, and these processes might be obstacles when scaling up the manufacturing technique to produce sufficient quantities of sterile material for clinical trial and, ultimately, commercialization [11].

This chapter encompasses investigations made progressively on the design of injectable peptide/protein-loaded PLGA microspheres. It covers an update on the state of art of the manufacturing of peptide/protein-loaded microspheres through both conventional and newer microencapsulation techniques, different analytical methods used for microsphere characterization, immune system interaction with microspheres following parenteral administration, and potential application of microspheres having therapeutic peptides/proteins. Special emphasis is given particularly on various instability problems and investigated mechanistic ways to obviate the possible instability problems of peptide/protein drug during microsphere preparation as well as its release from the microspheres. It should be added that although the chapter focuses mainly on PLGA microspheres, many of the destabilization mechanisms and stabilization approaches described herein can be valid to some extent for other polymeric delivery systems, too.

TABLE 2 Injectable Peptide/Proteins/Vaccines Encapsulated in Biodegradable Microspheres

Peptides, Protein, Vaccine	Technique	Polymer	Reference
<i>Vaccine</i>			
SPf 66 malaria vaccine	Double emulsion	PLGA	28
Multivalent vaccines of <i>Haemophilus influenzae</i> type b (Hib), diphtheria toxoid (DT), tetanus toxoid (TT), pertussis toxin (PT)	Spray drying	PLGA	29
Rotavirus	Double emulsion	PLG	30
<i>Polypeptides and Proteins</i>			
Insulin	Double emulsion	PLA polyethylene glycol (PEG)	12
Recombinant human epidermal growth factor (rhEGF)	Double emulsion	PLA	13
Ribozyme	Double emulsion	PLA, PLGA	14
Vapreotide (somatostatin analogue)	Spray drying	PLGA	15
Insulinlike growth factor-1 (IGF-1)	Double emulsion	PLGA-PEG	16
Ornithide acetate leuteinizing hormone releasing hormone [(LHRH) antagonist]	Dispersion/solvent extraction/evaporation	PLA, PLGA	17
Vascular endothelial growth factor (VEGF)	Single emulsion	PLGA/PEG	18
Human chorionic gonadotropin (hCG)	Double emulsion	PLA, PLGA	19
Calcitonin	Double emulsion	PLGA	20
FITC-bovine serum albumin (BSA)	Double emulsion	Poly(ϵ -caprolactone)	9
Levonorgestrel and ethinylestradiol	Double emulsion	Poly(ϵ -caprolactone)	10
Recombinant human bone morphogenetic protein	Double emulsion	PLGA	21–24
Transforming growth factor beta	Double emulsion	PLGA or PLGA-PEG	25–27
Recombinant human erythropoietin (rhEPO)	Modified double emulsion	LPLG-PEO-LPLG	31
Protein-C	Double emulsion	PLA	32
Ovalbumin	Double emulsion	PLGA	33
Human serum albumin	Double emulsion	PLA	34
Bovine serum albumin	Nonaqueous oil-in-oil (o/o) emulsion	PLG	35

Abbreviations: FITC: fluorescein isothiocyanate; PLG: poly(lactide-co-glycolide); LPLG-PEO-LPLG: copoly(l-lactic-co-glycolic acid-b-oxyethylene-b-l-lactic-co-glycolic acid); PEO: polyethylenoxide.

5.2.2 PEPTIDE/PROTEIN-LOADED MICROSPHERE PRODUCTION METHODS

The development of delivery systems for therapeutic peptides/proteins depends on biophysical, biochemical, and physiological characteristics of these molecules, including molecular size, biological half-life, immunogenicity, conformational stability, dose requirement, site and rate of administration, pharmacokinetics, and pharmacodynamics [36]. Unlike conventional drug molecules, the unique conformational structure of peptidic/proteinic therapeutic agents poses a great challenge right from the beginning of the selection of suitable microencapsulation techniques to make microspheres. Table 3 lists the considerations to be taken before choosing a particular encapsulation technique. Apart from the traditional phase separation technique, other techniques suitable for peptide/protein-loaded microsphere production can be divided into two main categories: during microsphere preparation, those involved in utilizing a hydrous environment such as emulsion-based methods and those based on an anhydrous environment such as spray freeze drying, spray drying, freeze drying, grinding, jet milling, liquid-phase antisolvent precipitation, and supercritical CO₂-based methods [37–40]. In the following sections, the various production techniques to make injectable peptide/protein-loaded microspheres are briefly introduced; however, a detailed discussion is beyond the scope of this chapter.

5.2.2.1 Phase Separation (A Traditional Technique)

Polymer phase separation or coacervation is an excellent technique for the encapsulation of water-soluble drugs including peptide/protein into a final microsphere product [41]. The peptide/protein molecule is dispersed in solid form into solution containing dichloromethane and PLGA. Silicone oil is added to this dispersion at a defined rate, reducing the solubility of polymer in its solvent. The polymer-rich liquid phase (coacervate) encapsulates the dispersed peptide/protein molecules and embryonic microspheres are subjected to hardening and washing using heptane. The process is quite sensitive to polymer properties, and residual solvent is also an important issue. Decapeptyl [triptorelin, a luteinizing hormone releasing hormone (LHRH) analogue] [42] and Somatuline LA (lanreotide, a somatostatin analogue) [43] are microsphere commercial products developed by this technique (Table 1).

TABLE 3 Factors in Selection of Microencapsulation Method to Prepare Peptide/Protein-Loaded Microspheres

Optimal peptide loading
High microsphere yield
Batch content uniformity
Interbatch reproducibility
Peptide stability during preparation and release
Size uniformity
Adjustable release profile
Low burst release
Flowability of final product
Residual solvent and polymer monomer control
Sterilization (both aseptic and terminal)

5.2.2.2 Double Emulsion (A Hydrous Technique)

Oil-in-water (o/w) and water-in-oil-water (w/o/w) are the two hydrous techniques representing respectively the single- and double-emulsion formation during microsphere preparation. However, the w/o/w technique is most commonly employed [44]. In this process, peptides/proteins in aqueous solution are emulsified with nonmiscible organic solution of polymer to form a w/o emulsion. Dichloromethane serves as organic solvent and the o/w primary emulsion is formed using either high-speed homogenization or ultrasonication. This primary emulsion is then rapidly transferred to an excess of aqueous medium containing a stabilizer, usually polyvinyl alcohol. Again homogenization or intensive stirring is necessary to initially form a double emulsion of w/o/w. Subsequent removal (by evaporation) of organic solvent by heat, vacuum, or both results in phase separation of polymer and core to produce microspheres. Instead of solvent evaporation, solvent extraction with a large quantity of water with or without a stabilizer can also be undertaken to yield microspheres containing peptide/protein. Although the w/o/w microencapsulation technique seems to be conceptually simple to carry out, the particle formation process is quite complicated, and a host of process parameters influence the properties of peptide/protein-loaded PLGA microspheres [45]. In spite of that, different peptides and proteins such as bovine serum albumin (BSA) or ovalbumin (OVA), insulin, recombinant human insulinlike growth factor-1 (rhIGF-1), recombinant human epidermal growth factor (rhEGF), human chorionic gonadotropin (hCG), protein C, recombinant human bone morphogenetic protein (rhBMP), and calcitonin, along with antigens and other therapeutically relevant proteins such as recombinant human erythropoietin (rhEPO), have been successfully encapsulated (see Table 2) by the w/o/w double-emulsion technique. Lupron Depot/Enantone Depot/Trenantone/Enantone Gyn (all having leuprolide acetate, a LHRH analogue) are very popular commercial microsphere products produced by this technique [46, 47], available both in the EU and United States, for the treatment of either prostate cancer of man or infertility (endometriosis) of women (Table 1).

5.2.2.3 Spray Drying (An Anhydrous Technique)

Spray drying is a rapid, convenient technique which can be conducted under aseptic conditions. First, a polymer—prevalently PLGA is applied—is dissolved in a volatile organic solvent such as dichloromethane or acetone. The protein is suspended as a solid or emulsified as aqueous solution in this organic solution by homogenization. After that, the resulting dispersion is atomized through a (heated) nozzle into a heated airflow. The organic solvent evaporates, thereby forming microspheres with dimensions of typically 1–100 μm . The microspheres are collected in a cyclone separator. For the complete removal of the organic solvent, a vacuum drying or lyophilization step can follow downstream.

The internal structure of the resulting polymeric microspheres depends on the solubility of the peptide/protein in the polymer before being spray dried leading to the formation of reservoir- or matrix-type products (see Figure 1). When the initial dispersion is solution, the final product obtained following spray drying is matrix or monolithic type, that is, polymer particles with a dissolved or dispersed nature of the active ingredient (defined as microspheres). Conversely, when the initial

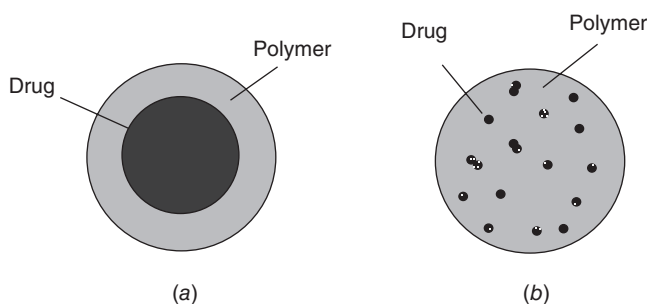


FIGURE 1 Polymeric delivery systems: (a) reservoir systems (microcapsules); (b) matrix systems (microspheres).

dispersion is in suspension, the product obtained is reservoir type, that is, a distinct polymeric envelope/shell encircling a liquid core of dissolved active ingredient (defined as microcapsules). Recombinant human erythropoietin [48] and bromocriptine mesylate, Parlodel Depot [49], are examples of microspheres (matrix type) obtained by the spray drying technique.

5.2.2.4 New Trends in Production Methods

Several issues such as reducing cost, reducing scale-up difficulties, improving protein stability, allowing for terminal sterilization, and eliminating the need for organic solvents during addition of the peptide/protein motivate the development of new methods to manufacture microspheres. Moreover conventional microencapsulation methods involve relatively harsh conditions that are not generally tolerated by peptide/protein molecules without stabilization. Therefore, new and improved processes shielding the peptide/protein from deleterious conditions have been proposed and evaluated.

Modified Conventional Methods The w/o/w solvent evaporation or extraction is probably one of the most widely used methods for peptide and protein microencapsulation [44], despite its many drawbacks. Improvements and alternatives have therefore been proposed such as oil in water (o/w), *o/w (the asterisk including cosolvent) and oil in oil (o/o) [50].

Utilising a modified w/o/w method, the rhIGF-1 was encapsulated into PLGA microspheres after increasing the pH of the protein solution from 4.5 to 5.5–6.0, where rhIGF-1 formed a viscous gel [51]. High entrapment efficiency of fully bioactive protein was achieved, and 92–100% of pure, monomeric, and bioactive rhIGF-1 was released in vitro over 21 days. The lowering of the rhIGF-1 solubility at pH 5.5–6.0 probably restricted its conformational flexibility and changes upon exposure to the polymer solvent. Without pH adjustment, approximately 10–32% of rhIGF-1 was lost upon solvent exposure, due to degradation and aggregation. Elsewhere, a w/o₁/o₂ system was investigated for encapsulating different proteins and peptides, with the o₁ and o₂ phases consisting of acetonitrile/dichloromethane and liquid paraffin/Span 80, respectively [52]. The acetonitrile mediated the partial mixing of the w and o₁ phases and subsequent protein/peptide precipitation, which was a

prerequisite for microencapsulation. The proteins BSA, tetanus toxoid (TT), and lysozyme did precipitate at low acetonitrile concentration, resulting in efficient microencapsulation (more than 90%), while a decapeptide and a linear gelatine did not precipitate so rapidly, resulting in poor entrapment. TT and lysozyme released during the burst phase (15%) maintained their bioactivity, although lack of further release suggested aggregation within the microspheres.

Another approach consisted of dispersing the protein antigen in a mineral oil before encapsulation into PLGA microspheres by a $o_1/o_2/w$ method [53]. The mineral oil (o_1) was intended as a barrier to protect the antigen during emulsification with the polymer solution and from exposure to moisture during release. Over 92% of enzyme-linked immunosorbent assay (ELISA) reactive TT was released from the reservoir-type microspheres in a pulsatile pattern, proceeding with an initial burst and followed by a second release pulse between 14 and 35 or 35 and 63 days, depending on the polymer type used. The latter stage of release was ascribed to TT diffusion through the oily phase, once an appreciable loss of polymer mass had occurred. The authors claimed the mineral oil was the key to protecting the solid antigen during polymer erosion, where acidic degradants and moisture would otherwise have led to antigen inactivation.

To improve solvent extraction, a novel method using a static micromixer was presented where a w_1/o dispersion (aqueous BSA in organic PLGA solution) is fed into an array of microchannels and the extraction fluid (w_2) into a second array of interdigitated channels [54]. The two fluids, transported separately through the channels, are discharged through an outlet slit where alternating fluid lamellae are formed with the w_1/o fluid lamella disintegrating into microdroplets, which harden quickly to form microspheres. This process offers easy scale-up, methodological robustness, continuous production, and a simple setup, making it ideally suited for aseptic production, a strongly needed feature for microsphere vaccine formulations.

ProLease Technology (Cryogenic Spray Drying) A variation of the conventional spray drying method is a cryogenic method which will be described below. A novel low-temperature spraying technique (called ProLease technology) for preparing PLA and PLGA microspheres has been reported by Khan et al. [55] and the group at Alkermes [56, 57]. The method relies on the use of stabilizing and release controlling agents, low processing temperature, and nonaqueous microencapsulation. Typically, a protein powder is micronized, possibly with a stabilizer, by spray freeze drying and then suspended in an organic polymer solution. The suspension is atomized into a vessel containing liquid N_2 overlaid by frozen ethanol (extraction solvent). The atomized droplets freeze in the liquid N_2 and deposit on the surface of the frozen ethanol. As liquid N_2 evaporates, the frozen ethanol liquefies (T_m approximately -110°C) so that the frozen polymeric droplets will transfer into the ethanol where the polymer solvent is extracted, yielding solid microspheres [58, 59]. To date, the ProLease system has been effectively applied to the encapsulation of zinc-complexed human growth hormone in PLGA microspheres, resulting in a one-month effect after one single injection [37, 57, 60]. As a reference, the recombinant human growth hormone (rhGH) was unstable in contact with ethyl acetate or dichloromethane [61]. The only protein-containing PLGA microspheres, Nutropin Depot, is produced by this novel technique. However, this product containing rhGH

marketed initially in the United States in 1999 was pulled from the market voluntarily by the manufacturer in June 2004 because of high costs of production and commercialization (<http://www.gene.com/gene/news/press-releases/>, accessed May 25, 2006).

ProLease technology was also used for encapsulating recombinant human vascular endothelial growth factor (rhVEGF) and rhIGF-1 [62, 63]. Both proteins were stabilized in aqueous solution, prior to spray freeze drying, and encapsulated (9–20% w/w) into PLGA microspheres. The microspheres also contained ZnCO_3 (3–6% w/w) as release modifier. The resistance of rhIGF-1 to aggregation and oxidation, determined from in vitro release studies, hardly changed. Protein, released in an almost pulsatile fashion over 21 days, was composed of predominantly monomeric rhIGF-1 with only minor amounts (~6%) of degradants forming toward day 21. Similarly, the integrity of rhVEGF dimer released over 21 days was good and its bioactivity remained largely unaffected, regardless of the extent of aggregation and degradation. In view of these studies, ProLease technology appears to have potential for sustaining antigen stability and release from microspheres.

Techniques Using Supercritical Fluids Generally, the application of supercritical (SC) fluids for the encapsulation of peptides and proteins has been fueled by the recognition that the established methods implicate some drawbacks. The application of supercritical fluids, especially of supercritical carbon dioxide (CO_2), can minimize or even eliminate the use of organic solvents and renders work at moderate temperatures possible [64]. The term *supercritical* defines the area above the critical point, which specifies the final point of the liquid–gas phase transition curve. Beyond that critical point, isobar/isotherm alterations of pressure or temperature alter the density of the critical phase but do not lead to a separation into two phases. A density change is directly associated with a change of the solvent power, and thus the method features a high variability. Usually CO_2 is used as supercritical fluid due to its critical point ($T_c = 31.1^\circ\text{C}$, $P_c = 73.8$ bars), which can be easily reached. That allows a moderate working temperature and leaves no toxic residues since it returns to the gas phase at ambient conditions. Two SC CO_2 -based processes have been reported for the preparation of drug-loaded polymeric microspheres: first, the rapid expansion from supercritical solutions (RESS) process, whereby a SC CO_2 solution of an active agent and a polymeric carrier is rapidly expanded. This quickly transforms the SC CO_2 into a liquid that is a much poorer solvent, thereby precipitating the active agent–carrier mixture as small particles [65]. Second is the aerosol solvent extraction system (ASES), also referred as the gas antisolvent spray precipitation (GAS) process [66]. Here, a solution of the active agent and the polymeric carrier is sprayed into a chamber loaded with SC CO_2 . The SC CO_2 extracts the solvent from the spray droplets and induces coprecipitation of the active agent and the polymeric carrier in the form of small, solvent-free particles [67, 68]. However, the use of organic solvents cannot be avoided, which is to be deemed a major disadvantage of both techniques.

In peptide/protein pharmaceuticals, the GAS process is predominantly applied for the preparation of microparticulate protein powders as an alternative to common drying processes. However, Winters et al. [69] reported an increase of β -sheet aggregates during the precipitation of lysozyme, trypsin, and insulin as a consequence of stress parameters such as organic solvent, pressure, and shear forces. One reason

why these methods were not credited as encapsulation techniques for protein within PLGA may be the tendency of several polymers to rapidly precipitate and agglomerate during the process [70].

ASES has been compared with conventional spray drying in terms of effects on the stability of the peptide tetracosactide [71]. Almost no intact peptide was recovered from spray-dried PLA particles, whereas the tetracosactide was well protected against oxidation during ASES (~94% unmodified peptide). In general, the particle formation step seems to be less detrimental to proteins than the loading step. For example, emulsification in an aqueous phase or spray drying of rhEPO/PLGA emulsions was mild compared to the first emulsification step [72]. Also, variation of the particle formation step (spray drying or coacervation) had a minor impact on diphtheria toxoid (DTd) antigenicity when compared to other process variables [73].

A serious limitation of GAS, ASES, and RESS for producing microspheres is the need of polymer types that form discrete crystalline domains upon solidification, such as l-PLA [74, 75]. The advantages of these methods offer (e.g., over spray drying) are the low critical temperatures for processing (34°C) and the avoidance of oxygen exposure during atomization, with both parameters being potentially important to peptide/protein stability.

Ultrasonic Atomization Ultrasonic atomization of w/o dispersions is presently under investigation for preparing especially protein antigen containing microspheres. In one setup, the atomized antigen/polymer dispersion was sprayed into a nonsolvent where the polymer solvent was extracted, resulting in microspheres [76]. A comparable technique was proposed where the antigen or polymer dispersion was atomized into a reduced pressure atmosphere and the preformed microspheres hardened in a collection liquid [77]. Similarly, PLGA solutions were also atomized by acoustical excitation and the atomized droplets transported by an annular stream of a nonsolvent phase [aqueous polyvinyl alcohol (PVA)] into a vessel containing aqueous PVA [78]. Solvent evaporation and microsphere hardening occurred in the vessel over several hours. The main advantages of these atomization techniques encompass the possibility of easy particle size control and scale-up, processing at ambient or reduced temperature, and the suitability for aseptic manufacturing in a small containment chamber such as an isolator.

In Situ Formed Injectable Microspheres All the encapsulation techniques discussed so far rely on the preparation of solid microspheres. However, a method for preparing a stable dispersion of protein containing semisolid PLGA microglobules has been reported [79]. Here, a protein dissolved in PEG 400 was added to a solution of PLGA in triacetin or triethyl citrate. This mixture, stabilized by Tween 80, was added dropwise and under stirring to a solution of Miglyol 812 or soybean oil, containing Span 80, resulting in a stable dispersion of protein inside semisolid PLGA microglobules. The microglobules remained in an embryonic state until mixed with an aqueous medium, so that the water-miscible components were extracted and protein containing matrix-type microspheres formed. Myoglobin was encapsulated and found to remain physically unchanged (circular dichroism analysis) after the process and during storage of the microglobular dispersion (15 days, 4°C).

Preformed Porous Microspheres A new approach for attaining sustained release of protein is introduced involving a pore-closing process of preformed porous PLGA microspheres [80]. Highly porous biodegradable PLGA microspheres were fabricated by a single w/o emulsion solvent evaporation technique using Pluronic F127 as an extractable porogen. The rhGH was incorporated into porous microspheres by a simple solution dipping method. For its controlled release, porous microspheres containing rhGH were treated with water-miscible solvents in the aqueous phase for production of pore-closed microspheres. These microspheres showed sustained-release patterns over an extended period; however, the drug loading efficiency was extremely low. To overcome the drug loading problem, the pore-closing process was performed in an ethanol vapor phase using a fluidized-bed reactor. The resultant pore-closed microspheres exhibited high protein loading amount as well as sustained rhGH release profiles. Also, the released rhGH exhibited structural integrity after the treatment.

Charged (Anionic and Cationic) PLGA Microspheres PLGA or any other type of microspheres can be readily decorated with positive or negative surface charges by simply preparing the particles by a w/o/w solvent evaporation/extraction process where the second water phase contains a cationic emulsion stabilizer [hexadecyltrimethylammonium bromide; poly(ethyleneimine); stearylamine] or an anionic emulsifier [sodium dioctyl-sulfosuccinate; sodium dodecyl sulfate (SDS)]. Such compounds attach tightly to PLGA surfaces during preparation and provide the necessary surface charge for ionic adsorption of counterions. It is known that a protein's surface charge depends on its isoelectric point (pI) and the pH of the medium in which it is dispersed. The use of particles with ionic surface charge offers several advantages over classical microencapsulation, among which the mild conditions for loading are probably the most attractive. PLGA microspheres with surface-adsorbed protein antigens and DNA have been highly efficient in inducing strong immune responses, as reviewed by Singh et al. [81] and Jilek et al. [82]. Nonetheless, it remains to be shown whether such particles are also suitable for eliciting long-term immunity after one or two injections.

Jabbal-Gill et al. [83, 84] noted the tendency for microencapsulated protein antigens to distribute heavily at the surface of PLGA microspheres and developed polymeric lamellar substrate particles (PLSP) by precipitating a highly crystalline poly(*L*-lactic acid)/organic solvent solution with water, followed by removal of remaining organic solvent with nitrogen purge. The particles, which can be sterilized by gamma irradiation and stored as a suspension for several months without changes to antigen absorption [84], possessed a large lamellar surface area and highly negative zeta potential (-35 to -42 mV) and could adsorb significant amounts of antigen (up to $50\mu\text{g}/\text{mg}$ microspheres) depending on pH, ionic strength, antigen-polymer ratio, and other factors. Release of protein antigen (TT) could be extended to over 1 month with minimal antigenic losses in released antigen, although most of the antigen was lost to the initial burst or to apparent irreversible adsorption (as indicated by the absence of reaching 100% release). Elevated antibody responses in mice were elicited using PLSP similar to one dose of aluminum adjuvant following subcutaneous administration of OVA at elevated doses (100 or $300\mu\text{g}$). Both immunoglobulin IgG1 and IgG2a antibody subtypes were of similar magnitude over 28 days in the PLSP/OVA groups, and cellular immunity was also observed following

immunization with a 38-kDa protein antigen against tuberculosis [85]. Similarly, Kazzaz et al. [86] created anionic PLGA microparticles by substituting the standard nonionic emulsifier PVA with anionic SDS during microsphere preparation. In addition to eliciting elevated antibody responses in mice relative to the soluble antigen, the adsorbed antigen elicited a potent cytotoxic T-cell (CTL) response, similar to that observed after infection from virus expressing the p55 gag and polymerase proteins. Moreover, the CTLs were formed from the more challenging intramuscular route but not significantly by the soluble antigen, even at elevated doses. The SDS-PLGA particles could also be gamma irradiated before adsorption and were shown to effectively boost antigen in nonhuman primates [87].

5.2.3 ANALYTICAL CHARACTERIZATION OF PEPTIDE/PROTEIN-LOADED MICROSPHERES

An area requiring additional efforts is analytical characterization of peptides and proteins encapsulated in PLGA microspheres. The high complexity of the therapeutic peptides and proteins requires not only physicochemical methodologies but also immunochemical and biological techniques for the characterization and quality control of these substances. In general, the analytical methods can be broadly viewed from the following study perspectives: methods meant for microsphere product quality checking, methods used for peptide/protein stability identification inside the microspheres, and methods called for peptide integrity detection following liberation from the microspheres immediately upon placement in release medium either *in vitro* or *in vivo*. Therefore, in most cases, a combination of several analytical methods is necessary for a comprehensive characterization of the peptide/protein under investigation and for appropriate quality control of the product concerning identity, purity, and potency. However, some of the analytical methods have potentially appealing applications to interplay among the mentioned perspectives. In Table 4, a selection of widely used analytical methods is given, showing which technology is applicable for the testing of identity, purity, and potency of peptides and proteins. In addition, peptide/protein integrity evaluation is indeed likely to be affected by artefacts during the sample preparation before analysis and during the analysis itself. Therefore, artefacts might prevent the scientist from critically ascribing detected protein denaturation to manufacturing conditions [88].

In order to measure the extent of peptide/protein degradation within the carriers and during release, the encapsulated molecule has to be removed from the polymeric matrix. Moreover, for avoiding artefacts such as underestimation of drug content, recovery methods need to be tried by an empirical trial-and-error approach as each peptide/protein is different one from the other. Recovery methods so far reported include extraction-based method with the help of potentially deleterious organic solvents, hydrolysis of the polymer matrix with alkaline medium, dissolution of polymer matrix in an organic solvent, recovery of suspended insoluble protein by filtration [89], total protein quantification after complete digestion of carriers followed by amino acid analysis [90, 91], electrophoretic extraction of the protein using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [92, 93], and direct dissolution of both the polymer and the protein drug in a single liquid

TABLE 4 Analytical Methods for Characterization and Quality Control of Pharmaceutical Peptides and Proteins

Methods	Indicated Usage/Checking		
	Identity	Purity	Potency
<i>Physicochemical</i>			
Chromatography			
Reversed-phase high-performance liquid chromatography (HPLC, RP-1)	+	+	-
Ion exchange	-	+	-
Affinity	-	+	-
Size exclusion chromatography (SEC)	-	+	-
Spectroscopy			
Infrared spectroscopy	+	+	-
Raman spectroscopy	+	+	-
Fluorescence spectroscopy	-	+	-
Ultraviolet/visible (UV/VIS) spectroscopy	+	+	-
NMR spectroscopy	+	+	-
Mass spectrometry	+	+	-
Circular dichroism (CD)	+	+	-
Matrix-assisted light desorption ionization–time-of-flight (MALDI-TOF) mass spectrometry	+	+	-
Electrophoresis			
Capillary electrophoresis	-	+	-
SDS–polyacrylamide gel electrophoresis (PAGE)	+	+	-
Isoelectric focusing	-	+	-
<i>Immunochemical</i>			
Radioimmunoassay (RIA)	-	-	+
ELISA	-	-	+
Western blot	+	-	-
<i>Biological</i>			
In vivo assays	-	-	+
In vitro (cell culture) assays	-	-	+

Abbreviation: SDS: sodium do decyl sulfate.

phase containing water-miscible organic solvents such as acetonitrile or dimethyl-sulfoxide (DMSO) [94, 95].

Following successful recovery of peptide/protein molecule from the microspheres, a simple spectrophotometric method does not always allow discrimination between the monomeric protein form and its aggregates. However, HPLC might separate these species and thus provides more accurate qualitative data [96]. But HPLC cannot quantify exclusively the amount of active protein antigen, as is the case with ELISA techniques [97]. Nowadays, Fourier transform infrared (FTIR) spectroscopy has become a popular, noninvasive method, as it is able to characterize the secondary structure of entrapped proteins [26, 95, 98–101]. Only recently, the integrity of their primary structure was evaluated, thanks to a new matrix-assisted laser

desorption ionization–time-of-flight (MALDI-TOF) mass spectrometry method [94, 102]. The method was shown to require little sample material and only a simple dissolution of the carrier was needed prior to the analysis. The MALDI-TOF allowed elucidation of a new degradation pathway, that is, peptide acylation within PLGA carriers resulting from a chemical interaction between peptide and degraded polymer [102]. Moreover, the method was also useful for quantification, and it should be underlined that no interference from PLGA was detected during the measurements. For all the reasons cited above, mass spectrometry should be considered one of the most promising methods for protein analysis inside polymeric carriers including microspheres. Using erythropoietin as an example, an exploratory and elaborative discussion was made on the analytical techniques used for the characterization and quality control of pharmaceutical peptides and proteins [103]. A similar discussion was also done on the analytical techniques critical to (as a part of) the quality assurance after process changes of the production of a therapeutic antibody [104].

With an increasing level of sophistication in the design of new protein antigens and adjuvants (including polymer controlled-release systems), efforts both in the United States and the EU are underway to respond with more appropriate regulations [105–107]. For example, the Committee for Proprietary Medicinal Products (CPMP), the primary scientific body in EU regulatory matters, is currently updating its “notes for guidance,” which guide/direct industry and regulatory authorities on content and evaluation of marketing authorization applications for vaccines [105]. Early drafts of these updates include more rigorous guidelines for new non-aluminum-based adjuvants, including antigen stability requirements (see Sesardic and Dobbelaer [105] for a discussion). Similar discussions ongoing in the United States have attempted to standardize requirements of controlled-release parenterals [106, 107], including specifics regarding *in vitro* release assays and the need to account for 80% or more of the encapsulating agent during the release period.

5.2.4 IMMUNE SYSTEM INTERACTION WITH INJECTABLE MICROSPHERES

Since microspheres are capable of forming a drug depot, the encapsulated peptide or protein is being slowly released over days or months at the injection site. Interestingly, the size of microspheres plays an important role in immune response. Microspheres with sizes smaller than 10 μ m can be directly taken up macrophages (and dendritic cells) through a phagocytosis mechanism while sizes greater than 10 μ m need to undergo biodegradation before phagocytosis can occur [108]. It was shown that within a few days of intramuscular injection PLGA microspheres less than 10 μ m are completely engulfed in a thin layer of connective tissue and thus evidenced infiltration by macrophages as a consequence of wound-healing response to injected particles [109]. It is feasible that the influx of these macrophages may cause degradation of the encapsulated protein and available protein released in the vicinity of the microspheres. Furthermore, it has been suggested that these macrophages are capable of producing proteolytic enzymes [110], which may result in the release and circulation of altered, inactive, or immunogenic forms of the encapsulated peptide or protein.

On the other hand, degradation, protein antigen release, location, and antigen presentation of microspheres larger than 10 μ m are expected to be different from smaller ones. Larger microspheres can provide an extracellular depot for secondary immune responses by way of B-cell stimulation [111–113]. In both cases, upon administration of microspheres, a foreign-body response occurs resulting in an acute initial inflammation despite the excellent tissue compatibility and biodegradability properties of polymers such as PLGA. This initial inflammation is followed by the infiltration of small foreign-body giant cells and neutrophils [114]. These immune cells could consume the released peptide or protein and produce an immune response. However, if released protein is recognized as a self-protein (e.g., homologous), the probability of an immune response by these cells is reduced. It is therefore always essential to release the protein in its native conformation. The release of aggregated or denatured protein from the microspheres may, in fact, result in an unwanted immune response [115]. It should be added that systematic studies to explore the effects of tissue response on the bioavailability of incorporated peptide or protein drug have not appeared extensively in the literature, with a few exceptions as described below. Using a light microscopic technique, bumps containing residual amounts of microspheres were observed at the injection site two weeks after administration of TT-encapsulated PLGA microspheres to mice and guinea pigs [116]. These bump formations may be due to chronic reactions, long-term immunogenicity, and immunological priming of mice and guinea pigs against the injected polymeric microspheres. The immunogenicity of microsphere-encapsulated vaccines can be varied to some extent by changing the physicochemical properties of the microspheres, for example, size, surface properties, and release kinetics of the antigen from the microspheres [111]. An interesting review by Jiang et al. [117] details the various reports on the relationship between *in vitro* protein antigen stability and immunogenicity, modulation of cell-mediated immune responses, and different formulation approaches to achieve the appropriate immune response with microencapsulated vaccine antigens. There has been some debate, arising from some animal experiments, that the antigenicity does not directly correlate with immunogenicity. However, the stability of protein antigens is considered to play a significant role in the quality and magnitude of immune response for the controlled-release single-dose vaccines as degraded or nonantigenic proteins may not be able to provide a continuous boost for generation of protective levels of high-affinity antibodies.

5.2.5 EXCIPIENT INCLUSION: INJECTABLE PEPTIDE/ PROTEIN-LOADED MICROSPHERES

Peptide and protein molecules are highly prone to degradation mechanisms that can be divided into two classes: physical and chemical [118]. Whereas chemical degradation leads to the loss of the protein's primary structure through oxidation, deamidation, peptide bond hydrolysis, isomerization, disulfide exchange, and covalent aggregation, physical degradation refers to the changes in the higher order structure (secondary and above) mainly by noncovalent aggregation and precipitation. In particular, aggregates formation during the encapsulation process must be avoided because these aggregates always represent loss of therapeutic efficacy and

increased immunogenicity which can endanger the patient's health [119, 120]. The following few examples indicate the fragility of peptide and protein molecules due to physical or chemical degradation: Aggregation of insulin has been well characterized and depends on unfolding of the insulin molecules [121]; aggregation of lyophilized formulations of BSA, β -galactoglobulin, and glucose oxidase are attributed to disulfide interchange [118]; deamidation contributes to reduction in catalytic activity of lysozyme [122] and ribonuclease at high temperatures [123]; and peptide bond hydrolysis results in loss of activity of lysozyme when heated to 90–100°C [122]. A recent introduction to this list is formaldehyde-mediated aggregation pathway (FMAP) unique to formaldehyde-treated protein antigens such as TT [117, 124, 125].

The formulator of injectable microspheres for peptide and protein faces multiple challenges: (i) to maximize physical and chemical stability, (ii) to prolong biological half-life, (iii) to increase absorption, (iv) to decrease antigenicity, and (v) to minimize metabolism. Thus, it is quite obvious that the fabrication of peptide- and protein-loaded microspheres requires several kinds of excipients for effective stabilization or immobilization of encapsulated therapeutic molecules. Excipients of choice are included specifically for controlling protein degradation in microspheres due to (a) external and internal environmental changes, (b) manipulating the initial burst release, (c) preventing protein adsorption onto delivery devices, and (d) neutralizing the causative acidic microclimate formation due to the acids liberated by the biodegradable lactic/glycolic-based polymers. Therefore, it is generally best to find conditions to stabilize the protein before other aspects of the formulation, such as controlled-release characteristics, are optimized. Typically, the appropriate excipients for the protein under investigation are experimentally selected among various substances by screening. This tedious experimental screening is partly necessary due to the present inability to predict protein stability after addition of such excipients. Moreover, since individual entrapped peptides and proteins differ in terms of physicochemical properties and chemical/therapeutic function, each species is expected to demonstrate a different degree of sensitivity to stress and react differently to the same stabilization strategy. For example, a sugar, amino acid, or antacid excipient may be required to stabilize protein, each of which can increase water uptake in the polymer matrix leading to an increase in release rate. In the scenario in which controlled-release conditions are optimized before such a stabilizer has been identified, it is likely that upon addition of the new stabilizer the release kinetics may change enough to require reformulation. Certainly, there is a sharp contrast between encapsulating a highly water soluble protein [126] or a poorly soluble zinc–protein complex [37, 57]. Switching between these two cases would be expected to alter the requirements in the formulation necessary to attain the controlled-release function (e.g., low versus high polymer matrix permeability for the protein, respectively) because protein solubility in water may be important for any diffusion component of release. The principal stresses causing instability of encapsulated peptide/proteins in PLGA microspheres are elaborated in a book chapter [127] and in a journal publication [128]. This subject was again reexamined in a review based on new findings since the previous book chapter by the same author [129]. An interesting review from the same research group was published on the biodegradable PLGA microparticles for injectable delivery of vaccine antigens [117], where they focused on mechanistic approaches to improve the stability of PLGA-encapsulated protein antigens.

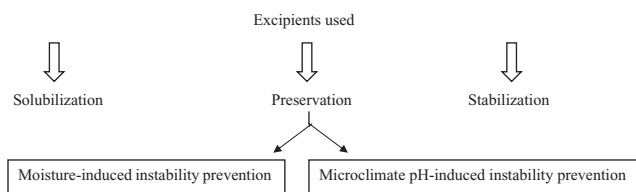


FIGURE 2 Flow chart of excipients used to prevent/minimize protein instability problems.

Another review by Bilati et al. [130] also envisioned the strategic approaches for overcoming peptide and protein instability within biodegradable nano- and microparticles. The reader is also referred to related publications edited by Sanders and Hendren [131] and Senior and Radomsky [132] for information on excipients used in injectable peptide/protein-loaded formulations including microspheres. This section will not cover all excipients used in parenteral protein formulations because the aforementioned publications already do so. Rather this section highlights examples of synergistic and antagonistic interactions that have been reported mainly between the excipients and the peptide/protein drugs, especially before microsphere preparation, followed by a brief discussion of a major instability problem of proteins/peptides inside the microspheres. The published research paper is being organized according to major functions of parenteral excipients, namely, solubilization, stabilization, and preservation (see Figure 2) [133].

5.2.5.1 Solubility- and Stability-Increasing Excipients

The traditional approach is to solubilize directly the peptide/protein in organic solvents. This can be achieved by different means. Cleland and Jones [61] assumed that native protein conformation could be maintained by precipitating the protein at its pI. The molecule is then free of charge and can be readily solubilized in organic solvents. Conversely, an alternative concept is based on the freeze drying of the protein at a pH away from its pI value before formulating it. It was thought that this strategy could increase protein solubility and stability in various polar and water-miscible organic solvents such as DMSO [134, 135]. It should be noted that a preformulation procedure consisting of using spray freeze drying with a suitable excipient was able to stabilize BSA before encapsulation by a nonaqueous method [35]. Using the dissolution approach, lysozyme was successfully formulated but incomplete lysozyme release from microspheres was observed and ascribed to aggregation [136]. Protein solubility can also be increased via an ion-pairing mechanism. The protein is modified by adding an oppositely charged surfactant that binds to the protein, so as to obtain a neutral hydrophobic entity and thus reduce direct contact between the protein and the organic solvent. Positively charged proteins and negatively charged surfactants should be employed, since cationic surfactants might have toxic side effects. This technique was shown to improve lysozyme conformational stability after a hydrophobic complex between lysozyme and oleic acid [137, 138]. A new interesting concept is to encapsulate an aggregated protein in a reversibly dissociable form in order to avoid the formation of irreversible aggregates during processing and to promote the sustained release of the native monomeric form. Growth hormone was successfully formulated with this approach [139].

Cyclodextrins (CD) have emerged as very effective additive compounds for solubilizing hydrophobic drugs. In the parenteral dosage form area, modified cyclodextrins such as hydroxypropyl- β -cyclodextrin and sulfobutylether- β -cyclodextrin have been reported to solubilize and stabilize many injectable drugs, including dexamethasone, estradiol, interleukin-2, and other proteins and peptides [140] without apparent compatibility problems [141]. In addition, CD-containing formulations (either 0.1 M sulfobutylether- β -cyclodextrin or 0.1 M hydroxypropyl- β -cyclodextrin) were shown to cause less damage to venous epithelial cells at the site of injection compared with formulations containing organic cosolvents [142]. When CD were coentrapped in the internal aqueous phase, erythropoietin (EPO) covalent aggregate formation was significantly reduced during microsphere preparation by the double-emulsion method [72] and lysozyme stability was improved [88]. Although the precise mechanism is unclear, interactions between amino acids and the hydrophobic inner cavity of CD may play a role [143]. However, CD showed no protecting effect on insulinlike growth factor-1 (IGF-1) [144] and hepatitis B core antigen (HBcAg) [145] and even promoted the loss of superoxide dismutase activity at high CD concentrations [146]. By contrast, carboxymethylcellulose (CMC) did not efficiently stabilize HBcAg and GH against dichloromethane-induced denaturation [61, 145]. Various types (α , β , and γ) of CD were examined for encapsulating TT in PLGA microspheres [147], with γ -hydroxypropyl-cyclodextrin effectively increasing TT encapsulation. However, CD also showed low efficiency in retaining spray-dried TT antigenicity, probably due to antigenic epitopes being buried inside the molecular CD core [147].

Surfactants have the ability to lower surface tension of protein solutions and prevent protein adsorption and/or aggregation at hydrophobic surfaces such as PLGA. Among them, nonionic surfactants are generally preferred as ionic surfactants might bind to groups in proteins and cause denaturation. Tween 20 was shown to greatly reduce the rate of formation of insoluble aggregates of recombinant human factor XIII caused by both freeze thawing and agitation stresses [148]. Maximum protection occurs at concentrations close to the critical micelle concentration of Tween 20, independent of initial protein concentration. In another report, Tween 20 at a 1% (w/v) concentration caused precipitation of a relatively hydrophobic protein (*Humicola lanuginosa* lipase) by inducing nonnative aggregates [149]. Similarly, nonionic surfactants such as Tween 20 or 80 were not good stabilizers for lysozyme and rhGH against the unfolding effect of the water–dichloromethane interface. It has been assumed that both the hydrophilic (PEG chains) and hydrophobic (fatty acid chain) parts of the polysorbate molecules were preferentially partitioned in the dichloromethane phase, leading to low protection efficacy [61, 98]. Exchange of Tween 20 for a less hydrophobic surfactant, PEG 3350, provided almost complete rhGH recovery irrespective of protein concentration. However, an opposing trend was seen with EPO encapsulation in PLGA microspheres [72]. Encapsulated protein aggregates increased (~15%) with different PEG types codissolved in the w_1 phase. Conversely, when three nonionic surfactants of different hydrophilic–lipophilic balances (HLBs) were coencapsulated with insulin by the w/o/w double-emulsion method, only Tween 20 was able to improve insulin stability within particles and to limit formation of high-molecular-weight products during the sustained-release period [150].

Tween 80 is well known to protect proteins against surface-induced denaturation [151]. Tween 80 was demonstrated to reduce hemoglobin aggregation in solution by preventing the protein from reaching the air–liquid interface or the liquid–surface interfaces [152]. Polyoxyethylene surfactants such as Tween 80 can form peroxide impurities after long-term storage. Knepp et al. [153] concluded that Tween 80 and other nonionic polyether surfactants undergo oxidation during bulk material storage and subsequent use and the resultant alkyl hydroperoxides formed can contribute to the degradation of proteins. In such formulations, they further reported that thiols such as cysteine, glutathione, and thioglycerol were most effective in stabilizing protein formulations containing peroxide-forming nonionic surfactants.

The Pluronics, also known as poloxamers (e.g., poloxamer 188, British Pharmacopoeia standard) are a well-studied series of commercially available, nonionic, triblock copolymers with a central block composed of the relatively hydrophobic poly(propylene oxide) flanked on both sides by blocks of the relatively hydrophilic poly(ethylene oxide) [154, 155]. The Pluronics possess an impressive safety profile and are approved selectively by the Food and Drug Administration (FDA) for pharmaceutical and medical applications, including parenteral administration [156]. The strong safety profile, commercial availability, ease of preparation, and well-studied physical properties make the Pluronics particularly appealing for drug delivery purposes. They have been used in several patented protein formulations as stabilizers and sustained-release injectables in development as solubilizing and stabilizing agents [157]. However, poloxamers, like Tweens, can form peroxide impurities over time. Poloxamer 188 was successfully used when mixed with PLGA for prolonged release of active interferon- α (INF- α) [158], but such a formulation had no effect on BSA secondary structure compared to PLGA alone [35]. Poloxamer 188 was not effective in preventing nerve growth factor (NGF) aggregation during in vitro release from microspheres generated by spray drying [159]. Complex interactions between poloxamer, BSA, and PLGA were believed to have influenced BSA microencapsulation [160]. The gelling property of the amphiphilic poloxamer 407 was successfully employed for urease encapsulation. The protein was likely protected during the microsphere preparation by a hydrated gelled structure due to the hydrophilic polyoxyethylene chains [161]. EPO aggregates in PLGA microspheres decreased when poloxamer 407 was incorporated at a level of 10% (w/w) [72].

Interleukin-1 α (IL-1 α) was protected by phosphatidylcholine from damage during the double-emulsion process but underwent inactivation during microsphere incubation [162]. Sodium dodecyl sulfate significantly reduced insulin aggregation at the dichloromethane–water interface, whereas dodecyl maltoside did not, this surfactant being more efficient at air–water or solid–water interfaces [163]. It should be mentioned that surfactants are used along with sugars, proteins, and polymers effectively for solubilization and stabilization purposes of peptide/protein in microspheres. Bilati et al. [130] give an overview on various proteins and polymers that act as stabilizing excipients during the development of peptide/protein-loaded microspheres.

5.2.5.2 Preservation-Imparting Excipients

Prevention/Minimization of Moisture-Induced Instability Moisture- and microclimate acid pH–induced instability (typically the aggregation) of the peptide/protein

encapsulated in PLGA microspheres has been monitored. Even several formulation strategies to inhibit these instability problems are being actively investigated. If the protein is expected to exist in the solid state within the PLGA polymer, the protein is remarkably prone to aggregation when formulated under conditions that allow moisture- and microclimate acid pH-induced instability. The two covalent aggregation mechanisms commonly described during exposure of the solid protein to moisture are the disulfide interchange/exchange [164] and the FMAP, which is operative for protein antigens that have been detoxified with formaldehyde exposure [124]. In the former pathway, the reaction is typically initiated by a thiolate ion on the protein or free thiolate ions that accompany β elimination of an intact disulfide [165]. Decreasing the concentration of the reactive species (e.g., lowering pH to favor the nonionized thiol, covalently blocking the thiol group, or oxidizing free thiols as they appear with divalent copper ion) has been shown to block this mechanism [165]. To inhibit the FMAP, strongly formaldehyde-interacting amino acids such as histidine and lysine [166] have been colyophilized with the formalinized protein antigen. On exposure to moisture, the amino acids appear to bind with the reactive Schiff base or equivalent electrophile [167] in the protein before a neighboring protein nucleophile can react to form an intermolecular cross-link [124]. Sorbitol has also been identified to inhibit the FMAP of TT at the maximal aggregating water content of the antigen, about 30 g H₂O/g protein [168], although whether this is a humectant effect [169] or a possible covalent reaction with the highly reactive electrophile in the antigen has not been determined. Several techniques have been developed to successfully bypass the destabilizing stress either by altering the role of water in the solid or immobilizing the protein or, alternatively, by directly inhibiting the aggregation. Clearly, one of the most significant findings in the field of peptide/protein stability in polymers is the success of the immobilization strategy of Zn²⁺ precipitation, as performed with human growth hormone [37, 57, 170, 171]. The 2:1 mole ratio Zn-protein complex, which immobilizes the rhGH as a solid precipitate in a near-native state [99], has been shown to confer superior stability on the protein encapsulated in PLGA for a one-month release incubation. Since then, other proteins such as INF- α [172] and NGF [173] were also stabilized in PLGA microspheres by this approach. Another interesting approach originating in the patent literature is the precipitation of erythropoietin with salting-in salt, ammonium sulfate [174], which is a technique commonly used in protein processing. Other methods to alter the role of water in the reaction involve the addition of agents that alter the amount of water sorbed in the polymer and/or the activity of the water present. For example, both water-soluble salts (NaCl) and antacid excipients (Mg(OH)₂) are known to dramatically increase the amount of water sorbed in PLGAs, with the former due to osmosis and the latter to a complex effect of neutralizing acidic degradation products and end groups of the polymer (which also involves an osmotic component) [126]. In contrast, for a given moisture content, humectants such as sorbitol, which dissolve in water bound to the protein, reduce the available free water necessary to mobilize the protein or perform other roles in deleterious reactions [169].

The alternative to bypassing the deleterious role of moisture is to inhibit the aggregation mechanism directly. Several ways to accomplish this have been reported, particularly in the solid state and in the absence of the polymer. Well-referenced and useful book chapters by Johnson [175] and Carpenter and Chang [176] are

available to thoroughly focus on the importance of making a lyophilized powder before loading the peptide or protein into an injectable microspheres. It has been stated that, in comparison to protein solution, the protein in the solid state would be less susceptible to shear forces that occur during an emulsification procedure or denaturation at oil–water interfaces. However, special precautions should be taken during freeze drying because the drying process itself will expose the protein to destabilizing stresses. To circumvent this problem, cryo- and lyoprotectants and bulking agents are usually included along with a peptide or protein solution while it undergoes the drying stages of the lyophilization process.

Cryo- and Lyoprotectants and Bulking Agents Various mechanisms are proposed to explain why excipients serve as cryo- or lyoprotectants. The most widely accepted mechanism to explain the action of cryoprotection is the preferential exclusion mechanism [177]. Excipients that will stabilize proteins against the effects of freezing do so by not associating with the surface of the protein. Such excipients actually increase the surface tension of water and induce preferential hydration of the protein. Examples of solutes that serve as cryoprotectants by this mechanism include amino acids, polyols, sugars, and polyethylene glycol.

For lyoprotection, that is, stabilization of proteins during the drying stages of freeze drying and during storage in the dry state, two mechanisms are generally accepted. One is the water substitute hypothesis [178] and the other is the vitrification hypothesis [179]. Both are legitimate theories, but both also have exceptions; that is, neither fully explain the stabilization of proteins by excipients during dehydration and dry storage [180]. The water substitute hypothesis states that a good stabilizer is one that hydrogen bonds to the protein just as water would do where it presents and, therefore, serves as a water substitute. Sugars are good water substitutes. (It may at first appear contradictory that sugars can serve both as cryoprotectants because of being excluded from the surface of the protein and as lyoprotectants that hydrogen bond to the protein. However, keep in mind that the excluded solute concept involves a frozen aqueous system whereas the water substitute concept occurs in a dry system.) This is why many freeze-dried protein formulations contain sucrose or trehalose. Nevertheless, during a w/o/w procedure to prepare peptide/protein-loaded PLGA microspheres, sugars are often added to the inner aqueous phase. Trehalose was shown to partially improve the BSA secondary-structure protection within PLGA microspheres and to facilitate BSA monomer release [26]. Trehalose and mannitol had a significant effect on the recovery of soluble nonaggregated interferon- γ (INF- γ) and rhGH after emulsification and ultrasonication [61], whereas no or very little protecting effect on IGF-1 against these stress factors was observed [144]. No effect of trehalose, mannitol, and sucrose was observed against o/w interface-induced degradation of lysozyme, whereas lactose and lactulose significantly improved its structural stability and activity, mostly if these additives were also added to the second aqueous phase [88, 100]. Lysozyme and trypsin activity was not improved by addition of sucrose, which was unable to protect them from an emulsion-induced denaturation and from sonication [98, 181]. Mannitol and sucrose dissolved together in the inner aqueous phase had slight effect on NGF activity [182] and neither mannitol nor lactose improved HBcAg immunogenicity during dichloromethane/water emulsification [145]. Surprisingly, sucrose and trehalose even decreased urease bioactivity, showing the opposite effect to that

expected [161]. Coencapsulation of maltose reduced α -chymotrypsin aggregation [183]. With respect to microspheres generated by spray drying, trehalose was effective in retaining TT antigenicity [147] and in preventing BSA secondary-structure degradation [35]. Trehalose protected efficiently NGF during the processing but did not prevent its aggregation during in vitro release [159].

The vitrification hypothesis states that excipients that remain amorphous (glass formers) form a glassy matrix with the protein with the matrix serving as a stabilizer. Acceptance of this hypothesis requires formulators to determine glass transition temperatures of formulations to be freeze dried and to develop freeze-dry cycles that maintain drying temperatures below the glass transition temperature. Reports are available to indicate that excipient stabilizers, which are capable of undergoing crystallization during storage, caused degradation (typically aggregation and loss of potency) of the protein [176, 184, 185].

Freeze-dried formulations typically contain one or more of the following bulking agents: mannitol, lactose, sucrose, trehalose, dextran 40, and povidone. These excipients may also serve as cryo- and/or lyoprotectants in protein formulations. Fakes et al. [186] studied these bulking agents for moisture sorption behavior before and after freeze drying. Moisture uptake certainly can affect drug stability in the freeze-dried state, particularly with peptides and proteins. When selecting a bulking agent, these properties, particularly the tendency for moisture uptake, must be considered by the formulation scientist in developing an optimally stable freeze-dried formulation. Several excipients can serve as stabilizers for proteins that are unstable during the drying phases of freeze drying and/or during long-term storage in the dry state. Typically, additives that will crystallize during lyophilization (e.g., mannitol) or will remain amorphous but unable to hydrogen bond to the dried protein (e.g., dextran) are not effective lyoprotectants for proteins. Excipients that will crystallize during freeze drying will also be relatively ineffective, as was shown with sucrose in *H. lanuginosa* lipase formulations [149]. However, these authors also reported that sucrose crystallization could be inhibited by decreasing the mass ratio of sucrose to protein and by minimizing the moisture content that serves to decrease the glass transition temperature during storage. The reverse can also be true for certain small molecules. For example, excipients (mannitol or sodium bicarbonate) that promoted the crystallization of cyclophosphamide during freeze drying stabilized the final product whereas excipients that did not allow crystallization (e.g., lactose) destabilized the final product [187]. Costantino et al. [188] studied the effects of a variety of parenteral excipients on stabilizing human growth hormone in the lyophilized state. Mannitol, sorbitol, methyl α -D-mannopyranoside, lactose, trehalose, and cellobiose all provided significant protection of the protein against aggregation, particularly at levels (131:1 excipient-to-protein molar ratio) to potentially satisfy water binding sites on the protein in the dried state. At higher excipient-to-protein ratios, mannitol and sorbitol crystallized and were not as effective in stabilizing the protein compared with low levels in which they remained in the amorphous, protein-containing phase.

Reducing sugars may not be as effective as other bulking agents, cryoprotectants, or lyoprotectants because they may potentially react with proteins via the Maillard reaction. For example, glucose will form covalent adducts with side-chain amino acids lysine and arginine of human relaxin [189]. In addition, a significant amount of serine cleavage from the C terminal of the B chain of relaxin was formed when

glucose was used as the excipient. These reactions did not occur if mannitol and trehalose replaced glucose in the lyophilized formulation. Lactose will react with primary amines in the well-known Maillard-type condensation reaction to form brown-colored degradation products [190]. Thus, lactose is known to be incompatible with amine-containing compounds such as aminophylline, amphetamines, and amino acids/peptides. This reaction occurs more readily with amorphous lactose than crystalline lactose.

Hydrophilic additives such as glucose are known to increase the porosity of microspheres, causing an increase in permeability to mass transport and a higher burst. However, a significant reduction in initial burst release of a highly water-soluble model peptide, octreotide acetate, from poly(D,L-lactide-co-glycolide) microspheres by the coencapsulation of a small amount of glucose (e.g., 0.2% w/w) was reported [191]. Using the double emulsion-solvent evaporation method of encapsulation, the effect of glucose on initial burst in an acetate buffer pH 4 was found to depend on polymer concentration, discontinuous phase/continuous phase ratio, and glucose content. Extensive characterization studies were performed on two microsphere batches, $\pm 0.2\%$ glucose, to elucidate the mechanism of this effect. However, no significant difference was observed with respect to specific surface area, porosity, internal and external morphology, and drug distribution. Continuous monitoring of the first 24-h release of octreotide acetate from these two batches disclosed that, even though their starting release rates were close, the microspheres plus glucose exhibited a much lower release rate between 0.2 and 24 h compared to those without glucose. The microspheres plus glucose showed a denser periphery and a reduced water uptake at the end of the 24-h release, indicating decreased permeability. However, this effect at times was offset as glucose content was further increased to 1%, causing an increase in surface area and porosity. In summary, these authors concluded that the effects of glucose on initial burst are determined by two factors: (1) increased initial burst due to increased osmotic pressure during encapsulation and drug release and (2) decreased initial burst due to decreased permeability of microspheres [191].

Mannitol is probably the most widely used bulking agent in lyophilized formulations because of its many positive properties with respect to crystallinity, high eutectic temperature, and matrix properties. However, some lots of mannitol can contain reducing sugar impurities that were implicated in the oxidative degradation of a peptide in a lyophilized formulation [192]. Mannitol at or above certain concentrations and volumes in glass vials is well known to cause vial breakage because of the unique crystallization properties of mannitol-ice during the primary drying states of freeze drying [193, 194].

Other Freeze-Dry Excipients High-molecular-weight carbohydrates such as dextran have higher glass transition temperatures than peptides/proteins. Therefore, when mixed with proteins, the overall glass transition temperature presumably can be increased with resultant increases in protein storage stability. Typically, carbohydrates (sucrose, trehalose, or dextran) alone do not result in appreciable increases in the storage stability of proteins. However, combinations of disaccharide and polymeric carbohydrates do tend to improve protein storage stability [195]. However, singular carbohydrates (sucrose or trehalose at 60 mM) were also just as effective in stabilizing a model recombinant humanized monoclonal antibody as combinations of sucrose and mannitol or trehalose and mannitol. Interestingly, with this

model monoclonal antibody, mannitol alone at 60mM provided less protection during storage than sucrose or trehalose alone. A specific sugar/protein molar ratio was sufficient to provide storage stability for this particular monoclonal antibody [196, 197].

Low-molecular-weight additives such as osmolytes (*N,N*-dimethylglycine, trehalose, and sucrose) or salts (sodium chloride, sodium phosphate, ammonium sulfate, and sodium citrate) were found to be highly effective in stabilizing keratinocyte growth factor, both against thermal denaturation and enhancing long-term storage stability [198]. Nevertheless, the stabilizing properties of osmolytes appear to be balanced between their binding to (deteriorating effect) and exclusion from (stabilizing effect) the peptide/protein surface. As binding or exclusion predominantly results from hydrophobic interactions, hydrogen bonding, and electrostatic interactions, the sum of the various interaction parameters are dissimilar for different proteins. Therefore, it becomes crucial to examine the individual nature of the additive toward each individual protein and to assess whether it will offer a stabilizing or destabilizing effect [199, 200].

Polyvinyl pyrrolidone (PVP) and glycine were found to stabilize lyophilized sodium prasterone sulfate whereas dextran 40 or mannitol did not [201]. PVP and glycine stabilized the pH of the reconstituted solution by neutralizing the acidic degradation product, sodium bisulfate, formed by the hydrolysis of prasterone sulfate. Dextran 40 or mannitol was ineffective because of no buffer capacity. Buffering agents, such as phosphate-citrate buffer and some neutral and basic amino acids (L-arginine, L-lysine, and L-histidine), also stabilized prasterone sulfate. L-Cysteine is an example of an amino acid that did not stabilize the drug, presumably because of its weak buffer capacity. Although the efficiency of proteinic additives for protein stabilization has been clearly demonstrated in several occasions even during encapsulation processes [31, 72, 98, 144], their use in pharmaceuticals is at present not desirable from a strictly regulatory point of view. Additionally, such agents might contribute to complicate all subsequent protein characterization within the formulation. Among these additives, albumins and gelatins are those mainly used for protection purposes. The protective effect of albumins against protein unfolding and aggregation has been extensively documented and is likely due to their surface-active properties (see Bilati et al. [130] for details).

Prevention/Minimization of Microclimate pH-Induced Instability Evidence for acidification within degrading microspheres is investigated and local pH values between 1.5 and 4.7 are being reported [202–204]. Methods to measure microclimate pH in PLGA microspheres include (i) ensemble average measurements using electron paramagnetic resonance (EPR) [203, 204], nuclear magnetic resonance (NMR) [205], and potentiometry and (ii) direct visualization techniques such as confocal imaging of pH-sensitive dyes [206, 207]. In the EPR method, the constant of hyperfine splitting, $2a_N$, was used to determine an average pH inside PLGA microspheres. Because the experiments relied on the mobility of spin-labeled protein, with an increase of the microviscosity in the later hours of the experiments, the spectra of EPR was changed and the signal-to-noise ratio decreased to prevent the measurement of pH throughout the release period [203]. The potentiometric measurements can give rapid values of pH for thin polymer films, and the pH of the thin water film between the electrode and polymer mimics the microclimate pH of aqueous pores inside the polymer-based drug delivery system. However, it is difficult to mimic

microclimate pH of a small-scale system, such as microspheres or nanospheres, which may have unique microstructures, excipient/drug distributions, and transport characteristics. Overall, the ensemble average measurements described above could give a general picture of microclimate pH at the macroscopic level. However, the microscopic level of the detection can only be achieved through direct visualization techniques, such as microscopic imaging. Shenderova et al. [207] first developed the confocal microscope imaging method to relate the microclimate pH with the fluorescent intensity. Because of the difficulty of controlling and predicting the fluorescein concentration in the aqueous pore inside the microsphere, the method was only semiquantitative. Fu et al. [206] improved the confocal microscopic imaging method by coencapsulating two dextran fluorescent dye (NERF and SNARF-1) conjugates inside microspheres and related the ratio of the two dye images with microclimate pH in order to eliminate the poorly controlled effects of dye concentration and pore distribution. However, both of the dyes emit in the green range (535 nm for NERF and 580 nm of SNARF), giving rise to poor resolution without a narrow-bandwidth detector. Because of the high noise-to-signal ratio from the ratio images, the prediction of pH is also expected to be semiquantitative. In order to overcome the aforementioned drawbacks in microclimate pH measurement, a new quantitative ratiometric method based on laser scanning confocal microscopic imaging was developed to create a pixel-by-pixel neutral range microclimate pH map inside PLGA microspheres [208]. This method was then applied to both acid-neutralized and nonneutralized PLGA microspheres during extended incubation in physiological buffer. In another study, the PLGA water-soluble acid distribution has been measured with prederivatization HPLC [209].

Ongoing acidification of the microsphere interior was shown to induce deamidation and covalent dimerization of nonreleased insulin [202]. Despite the evidence of acidification mentioned above, there is controversy on this subject. It has been pointed out that the sampling scheme has a significant impact on the degree of acidification; frequent replenishment of the release medium or the use of a dialysis bag can effectively prevent the acidification of the medium with subsequent reduced protein degradation [93, 210]. It is unsure, however, whether this also reflects the situation *in vivo*, in which the PLGA microspheres are often surrounded by a fibrous capsule that may reduce efflux of acidic degradation products from the PLGA matrix [93]. On the other hand, studies on rhGH-loaded PLGA microspheres showed a reasonable *in vitro*–*in vivo* correlation (IVIVC) only when a strong high-capacity buffer [200 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.4] was used, which effectively minimized the pH drop [211].

As indicated by the prevention of acid-induced physical aggregation of BSA in an abstract [212], three principal ways have been identified thus far to avoid the formation of highly acidic microclimate regions in the PLGAs during protein release:

- (i) Increasing the permeability of the polymer to facilitate escape of the water-soluble hydrolytic products of the PLGA polyester [125]
- (ii) Decreasing the degradation rate of the polyester [213]
- (iii) Coencapsulating additives to neutralize the weak acids formed by PLGA hydrolysis [126]

In addition, two more ways that are likely to favor a lowering of microclimate pH are elevated initial acid content in the polymer [214] and low-frequency release media exchange [206].

The concept of controlling polymer permeability is difficult because attempts to increase permeability can spoil the controlled-release function of the polymer and cause the encapsulated protein to be released too rapidly. For example, Jiang and Schwendeman [213] increased the permeability of slow-degrading PLA (molecular weight (MW) 145 kDa) by blending in PEG (MW 10 or 30 kDa) at 0, 10, 20, and 30%. Insoluble BSA aggregation in the PLA microspheres containing 4.5–5% w/w BSA was found in 0 and 10% PEG after a one-month incubation, but not in those preparations containing 20 or 30% PEG. The structural integrity of BSA was also intact in the stabilized formulations. However, between 10 and 30% PEG, the release rate of BSA increased rapidly and by 30% PEG, 60% of the protein encapsulated was released in only three days [213]. In contrast, an abstract [212] implied that 5% BSA encapsulated in a more permeable PLA (MW 77 kDa), the BSA formed <2% insoluble aggregates over one month. This result suggested strongly that in some instances the slow degradation rate of the non-glycolic-acid-containing PLA is sufficient to inhibit acid formation in the microclimate.

In instances in which it is desirable to increase permeability and/or decrease the hydrolytic rate of PLGAs, that is, where a highly water-soluble protein requires release for one month or longer, it becomes necessary to coencapsulate a basic additive. Antacids such as MgCO_3 , $\text{Mg}(\text{OH})_2$ or ZnCO_3 have been found to be particularly potent in preventing instability of acid-labile proteins [126, 215]. By means of thin films coating pH glass electrodes to measure directly the microclimate acidic environment in PLGA microspheres, the stabilization against insoluble acid-induced noncovalent BSA aggregation afforded by a series of antacid excipients has been correlated with the ability of the antacid to neutralize acidic pores in films of the same lot of PLGA coating pH glass electrodes [215]. Though much of the physical chemistry of microclimate pH adjustment with antacid additives is currently unclear, the strength of the base, the base solubility, and the association of the divalent cation with the carboxylate of the degradation products and/or polymer end groups appear to be important. For instance, Shenderova [216] has shown that from microclimate pH measurements in PLGA films coating pH glass electrodes, MgCO_3 and $\text{Mg}(\text{OH})_2$ were found to be very similar under conditions which favor homogenous neutralization (i.e., high protein loading sufficient to make pores for the base to diffuse all regions of the polymer matrix), but MgCO_3 was found to increase microclimate pH higher than $\text{Mg}(\text{OH})_2$. This result was consistent with the improved BSA stability in PLGA 50/50 microspheres when MgCO_3 was used in place of $\text{Mg}(\text{OH})_2$ [126].

5.2.6 PEPTIDE/PROTEIN ENCAPSULATED INTO BIODEGRADABLE MICROSPHERES: CASE STUDY

Selected examples of therapeutic peptide and protein including vaccines which have been encapsulated into biodegradable polymer-based microspheres are discussed in this section. Besides what is mentioned below, many other proteins and vaccines have been encapsulated in biodegradable polymers, so a glimpse of ongoing

research on microsphere delivery systems using biodegradable polymers is shown in Table 2.

5.2.6.1 Vaccines

Group B Streptococcus Vaccine Group B streptococcus (GBS) is the leading bacterial cause of neonatal sepsis and meningitis. Although antibiotic prophylaxis has decreased the infection rate, the best long-term solution lies in the development of effective vaccines. The GBS capsular polysaccharide (CPS) is a major target of antibody-mediated immunity. The feasibility of producing a GBS having the ability to produce both a local IgA immune response at the mucosal surface and humoral IgG response having capability of transplacental passive immunization was investigated [217]. Inactivated GBS antigen was encapsulated in PLGA by a w/o/w multiple-emulsion technique along with immunostimulatory synthetic oligodeoxynucleotides containing cytosine phosphate guanosine (CpG) as potent adjuvant [217]. Immunization of female mice with normal immune systems was done with these PLGA microspheres containing GBS type III polysaccharide and CpG adjuvant (PLGA/GBS/CpG) and results indicated a significantly higher GBS antibody response as compared to nonencapsulated GBS antigen or PLG-encapsulated GBS PS vaccine without the addition of the CpG.

Diphtheria Toxoid (DT) Diphtheria is a communicable disease caused by *Corynebacterium diphtheriae* which colonizes and forms a pseudomembrane at the infection site. This pathogen produces a potent protein toxin, diphtheria toxin, which is responsible for the typical systemic toxemia. DT is required for active immunization against diphtheria. DT was encapsulated in different types of PLA and PLGA microspheres by spray drying and coacervation. Immunization of guinea pigs with DT microspheres made with relatively hydrophilic PLGA 50:50 resulted in specific and sustained antibody responses to alum adjuvanted toxoid in contrast to microspheres made with hydrophobic polymers where very low antibody responses were determined confirming the feasibility of microsphere vaccines to induce strong, long-lasting protective antibody responses after single immunization [218].

In an endeavor toward development of multivalent vaccines based on biodegradable microspheres, Peyre et al. [219] tested the immunological performance of several divalent microsphere formulations against tetanus and diphtheria. Microspheres were made by separate microencapsulation of tetanus and diphtheria toxoid in PLGA by either spray drying or coacervation. Guinea pigs were subcutaneously immunized by a single injection of the divalent vaccines or, for control, an equivalent dose of a licensed vaccine containing both antigens adsorbed on aluminum hydroxide. All microsphere formulations were strongly immunogenic, irrespective of particle size and hydrophobicity. Endpoint titers of ELISA antibodies, mainly of the IgG1 subtype, were comparable to those obtained after immunization with the licensed vaccine. The microspheres provided increasing levels of antibodies, during the 16 weeks of testing, and the antibodies were weakly polarized toward tetanus. The induced antibodies were also toxin neutralizing, as determined for both diphtheria (1–4 IU/mL) and tetanus (5–9 IU/mL) eight weeks after immunization. These neutralization levels were several orders of magnitude above the level considered

minimum for protection (0.01 IU/mL). When the animals were challenged with tetanus or diphtheria toxins six weeks after immunization, microsphere vaccines produced protective immunity that was comparable to or better than that induced by the licensed divalent vaccine. In conclusion, this study showed that a single administration of biodegradable microsphere vaccines provided protective immunity against diphtheria and tetanus and that this immunization approach might be feasible for multivalent vaccines. In a separate study, the same group have studied for the first time the fate of immunogenic fluorescent-labeled PLGA microspheres loaded with DT in vivo following a subcutaneous injection route [220].

A unique instability problem of DT is being foreseen when the DT would be encapsulated in PLGA microspheres along with a preservative such as thiomersal [221]. Thimerosal (TM)—also known as thiomersal, Merthiolate, or sodium ethylmercuri-thiosalicylate—is a water-soluble derivative complex of thiosalicylic acid (TSA) that has been used as bactericide in parenteral vaccines and ophthalmic products for decades. It has been reported that this preservative can be decomposed by oxidation to 2,2-dithiosalicylic acid, ethyl mercuric ion, 2-sulfenobenzoic acid, 2-sulfobenzoic acid, and 2-sulfinobenzoic acid [222]. Namura et al. [221] demonstrated in vitro that the TSA, produced after the reduction of TM by lactic acid, reduces the S–S bridge of the previously incubated DT. This reduction is immediately followed by blocking the two SH groups formed by the same TSA molecules. In light of these conclusions, it is necessary now to reinterpret the in vitro protein degradation–stabilization data in the presence of PLGA microsphere, mainly for those proteins which contain S–S. The authors propose that all the PLGA microsphere microencapsulation studies and protein structural considerations should be done in the absence of TM as preservative.

Tetanus Toxoid (TT) Tetanus is considered a major health problem in developing and underdeveloped countries, with approximately one million new cases occurring each year. Tetanus is an intoxication manifested primarily by neuromuscular dysfunction. So vaccination is required for prevention of this disease. TT was encapsulated using PLGA with different molar compositions (50:50, 75:25) by the w/o/w multiple-emulsion technique and protein integrity was evaluated during antigen release in vitro in comparison to alum-adsorbed TT for in vivo induction of tetanus-specific antibodies [223]. TT microspheres elicited antibody titers as high as conventional alum-adsorbed TT, which lasted for 29 weeks, leading to the conclusion that TT microspheres can act as potential candidates for single-shot vaccine delivery systems.

The study by Determan et al. [224] focuses on the effects of polymer degradation products on the primary, secondary, and tertiary structure of TT, OVA, and lysozyme after incubation for 0 or 20 days in the presence of ester (lactic acid and glycolic acid) and anhydride [sebacic acid and 1,6-bis(*p*-carboxyphenoxy)hexane] monomers. The structure and antigenicity or enzymatic activity of each protein in the presence of each monomer was quantified. SDS-PAGE, circular dichroism, and fluorescence spectroscopy were used to assess/evaluate the primary, secondary, and tertiary structures of the proteins, respectively. ELISA was used to measure changes in the antigenicity of TT and OVA and a fluorescence-based assay was used to determine the enzymatic activity of lysozyme. TT toxoid was found to be the most stable in the presence of anhydride monomers, while OVA was most stable in the

presence of sebacic acid, and lysozyme was stable when incubated with all of the monomers studied.

Jaganathan et al. [225] compared the efficiency of microspheres produced from PLGA and chitosan polymers by using protein stabilizer (trehalose) and acid-neutralizing base $[\text{Mg}(\text{OH})_2]$. The immunogenicity of PLGA- and chitosan microsphere-based single-dose vaccine was evaluated in guinea pigs and compared with multiple doses of alum-adsorbed TT. Results indicated that a single injection of PLGA and chitosan microspheres containing TT could maintain the antibody response at a level comparable to the booster injections of conventional alum-adsorbed vaccines. Both the PLGA- and chitosan-based stable vaccine formulations produced an equal immune response. Hence chitosan can be used to replace the expensive polymer PLGA. This approach should have potential application in the field of vaccine delivery.

The study by Kipper et al. [226] focuses on the development of single-dose vaccines based on biodegradable polyanhydride microspheres that have the unique capability to modulate the immune response mechanism. The polymer system employed consists of copolymers of 1,6-bis(*p*-carboxyphenoxy)hexane and sebacic acid. Two copolymer formulations that have been shown to provide extended-release kinetics and protein stability were investigated. Using TT as a model antigen, *in vivo* studies in C3H/HeOuJ mice demonstrated that the encapsulation procedure preserves the immunogenicity of the TT. The polymer itself exhibited an adjuvant effect, enhancing the immune response to a small dose of TT. The microspheres provided a prolonged exposure to TT sufficient to induce both a primary and a secondary immune response (i.e., high antibody titers) with high-avidity antibody production, without requiring an additional administration. Antigen-specific proliferation 28 weeks after a single immunization indicated that immunization with the polyanhydride microspheres generated long-lived memory cells and plasma cells (antibody-secreting B cells) that generally do not occur without maturation signals from T helper cells. Furthermore, by altering the vaccine formulation, the overall strength of the T-helper type 2 immune response was selectively diminished, resulting in a balanced immune response, without reducing the overall titer. This result is striking, considering free TT induces a T-helper type 2 immune response and has important implications for developing vaccines to intracellular pathogens. The ability to selectively tune the immune response without the administration of additional cytokines or noxious adjuvants is a unique feature of this delivery vehicle that may make it an excellent candidate for vaccine development.

Poly(lactide) (PLA) polymer particles entrapping TT were evaluated in terms of particle size, antigen load, dose, and additional adjuvant for achieving high and sustained anti-TT antibody titers from single-point intramuscular immunization [227]. Admixture of polymer-entrapped TT and alum improved the immune response in comparison to particle-based immunization. High and long-lasting antibody titer was achieved upon immunization with 2–8- μm size microparticles. Microspheres within the size range 50–150 μm elicited very low serum antibody response. Immunization with very small particles (<2 μm) and with intermediate-size-range particles (10–70 μm) elicited comparable antibody response from single-point immunization but lower in comparison to that achieved while immunizing with 2–8- μm particles. Potentiation of antibody response on immunization of admixture of microspheres and alum was also dependent on particle size. These results indicate the need of

optimal particle sizes in micrometer ranges for improved humoral response from single-point immunization. Increasing antigen load on polymer particles was found to have a positive influence on the generation of antibody titers from particle-based immunization. Maximum peak antibody titer of $\sim 300\mu\text{g/mL}$ was achieved on day 50 upon immunization with particles having the highest load of antigen ($94\mu\text{g/mg}$ of polymer). Increase in dose of polymer-entrapped antigen resulted in concomitant increase in peak antibody titers, indicating the importance of antigen stability, particle size, and load on generating a reproducible immune response. Optimization of particle size, antigen load, dose, and use of additional adjuvant resulted in high and sustained anti-TT antibody titers over a period of more than 250 days from single-point immunization. Serum anti-TT antibody titers from single-point immunization of admixture of PLA particles and alum were comparable with immunization from two divided doses of alum-adsorbed TT.

Vibrio Cholerae (VC) Whole-Cell Vaccine Cholera, an acute intestinal infection caused by the bacterium *Vibrio cholerae*, produces an enterotoxin that causes a copious, painless, watery diarrhea that can quickly lead to severe dehydration and death if treatment is not promptly given. For prevention of cholera, cholera vaccine is usually given. VC was successfully entrapped in the PLGA microspheres by a double-emulsion method with trapping efficiencies up to 98%. The immunogenic potential of VC-loaded microspheres physically mixed with or without amphotericin B was evaluated in adult mice by oral immunization in comparison to VC solution. The immunogenicity of VC-loaded microparticles mixed with amphotericin B in evoking *Vibrio*-specific serum IgG and IgM responses was higher than that of VC-loaded microparticles only [228, 229]. However, VC was loaded in different polymer compositions (50:50 PLGA, 75:25 PLGA, and PLA/PEG blended), the higher antibody responses and serum IgG, IgA, and IgM responses were obtained when sera from both VC-loaded 75:25 PLGA and PLA/PEG-blended microparticles immunized mice were titrated against VC solution [230].

Japanese Encephalitis Virus (JEV) Japanese encephalitis is a disease that is spread to humans by infected mosquitoes in Asia. It is one of a group of mosquito-borne viral diseases that can affect the central nervous system and cause severe complications and even death. Vaccination is one of the ways of treating it. JEV vaccine was encapsulated in PLGA microspheres by a double-emulsion technique and influences of various process variables such as stirring rate, types and concentration of emulsifier, and polymer concentration were studied on size, size distribution, and biodegradation. The mean size of microspheres decreased with increasing speed, increasing concentration of emulsifier, and decreasing polymer concentration. Rate of biodegradation of nonporous microspheres was slower than that of porous microspheres, leading to the conclusion that PLGA microspheres can be used to apply oral vaccination through Peyer's patches across the gastrointestinal tract (GIT) [231].

Several approaches to develop an improved JEV vaccine are in progress in various laboratories. Of these, immunization of mice with plasmid DNA encoding JEV envelope (E) protein has shown great promise. The technology, developed by Kaur et al. [232], involved the adsorption of DNA onto cetyltrimethyl-ammonium bromide (CTAB) containing cationic poly(lactide-co-glycolide) (PLG) microspheres.

The microsphere-adsorbed DNA induced a mixed Th1–Th2 immune response as opposed to Th1 immune responses elicited by the naked DNA.

JEV-loaded poly(lactide) (PLA) lamellar and PLG microspheres were successfully prepared with low-molecular-weight PLA by the precipitate method and with 6% w/v PLG in the organic phase, 10% w/v PVP, and 5% w/v NaCl in the continuous phase by using a w/o/w emulsion/solvent extraction technique, respectively [233]. The JEV incorporation, physicochemical characterization data, and animal results obtained in this study may be relevant in optimizing the vaccine incorporation and delivery properties of these potential vaccine targeting carriers.

Hepatitis B Virus Hepatitis B is one of the most important infectious diseases in the world. Approximately 350 million people worldwide are chronic carriers of the hepatitis B virus (HBV), which accounts for approximately one million deaths annually. PLGA microspheres loaded with recombinant HBsAg were formulated using a double-emulsion technique. The pharmaceutical characteristics of size, surface morphology, protein loading efficiency, antigen integrity, release of HBsAg-loaded PLGA microspheres, and degradation of the polymer in vitro were evaluated [234–237]. Based on these findings in vitro and in vivo, it was concluded that HBsAg was successfully loaded into the PLGA microspheres, which can autoboot an immune response, and the HBsAg-loaded PLGA microsphere is a promising candidate for the controlled delivery of a vaccine.

5.2.6.2 Proteins

Prolidase Deficiency of this enzyme results in chronic intractable ulcerations of the skin, particularly of lower limbs, since it is involved in the final stages of protein catabolism. To counteract the problem, the enzyme was encapsulated in PLGA microspheres by a double- or multiple-emulsion technique, in vitro and ex vivo evaluations were done, and the results indicated that microencapsulation stabilizes the enzymatic activity inside the PLGA microspheres resulting in both in vitro and ex vivo active enzyme release, hence opening the doors for the possibility of enzyme replacement therapy through microencapsulation [238]. Further evaluation from the same research group for prolidase-loaded PLGA microspheres is reported elsewhere [239, 240].

Insulin Insulin is the most important regulatory hormone in the control of glucose homeostasis. The World Health Organization (WHO) has indicated that more than 50 million people around the world suffer from diabetes and require daily parenteral injections of insulin to stay healthy and live normally. For the treatment of type I diabetes insulin still is number one, with three subcutaneous injections to be taken per day. A controlled-release system for a long-term therapy of this disease is the need of the hour, as this can obviate the need for painful injection given a number of times to the diabetes patients. Insulin was encapsulated in blends of poly(ethylene glycol) with PLA homopolymer and PLGA copolymer by a w/o/w multiple-emulsion technique with entrapment efficiencies up to 56 and 48% for PLGA/ PEG and PLA/ PEG, respectively [12]. Insulin-loaded microspheres were capable of controlling the release of insulin for 28 days with in vitro delivery rates of 0.94 and 0.65 µg insulin/mg per particle per day in the first 4 days and steady release with a

rate of 0.4 and 0.43 μg insulin/mg per particle per day over the following 4 weeks, respectively, along with the extensive degradation of PLGA/ PEG microspheres as compared to PLA/ PEG blends which resulted in a stable particle morphology along with reduced fragmentation and aggregation of associated insulin.

Two types of injectable cationized microspheres were prepared based on a native gelatin (NGMS) and aminated gelatin with ethylenediamine (CGMS) to prolong the action of insulin [241]. Release of rhodamin B isothiocyanate insulin from CGMS was compared with that from NGMS under *in vitro* and *in vivo* conditions. Lower release of insulin from CGMS compared with that from NGMS was caused by the suppression of initial release. The disappearance of ^{125}I -insulin from the injection site after intramuscular administration by NGMS and CGMS had a biphasic profile in mice. Almost all the ^{125}I -insulin had disappeared from the injection site one day after administration by NGMS. The remaining insulin at the injection site after administration by CGMS was prolonged, with approximately 59% remaining after 1 day and 16% after 14 days. The disappearance of CGMS from the injection site was lower than that of NGMS. However, the difference in these disappearance rates was not great compared with those of ^{125}I -insulin from the injection site by NGMS and CGMS. The time course of disappearance of ^{125}I -CGMS from the injection site was similar to that of ^{125}I -insulin by CGMS. The initial hypoglycemic effect was observed 1 h after administration of insulin by NGMS, and thereafter its effect rapidly disappeared. The hypoglycemic effect was observed 2–4 h after administration by CGMS and continued to be exhibited for 7 days. The prolonged hypoglycemic action by CGMS depended on the time profiles of the disappearance of insulin from muscular tissues, which occurs due to the enzymatic degradation of CGMS.

A novel controlled-release formulation was developed with PEGylated human insulin encapsulated in PLGA microspheres that produces multiday release *in vivo* [242]. The insulin is specifically PEGylated at the amino terminus of the B chain with a relatively low molecular weight PEG (5000 Da). Insulin with this modification retains full biological activity but has a limited serum half-life, making microencapsulation necessary for sustained release beyond a few hours. PEGylated insulin can be codissolved with PLGA in methylene chloride and microspheres made by a single o/w emulsion process. Insulin conformation and biological activity are preserved after PEGylation and PLGA encapsulation. The monolithic microspheres have inherently low burst release, an important safety feature for an extended-release injectable insulin product. In PBS at 37°C, formulations with a drug content of approximately 14% show very low (<1%) initial release of insulin over one day and near-zero-order drug release after a lag of three to four days. In animal studies, PEG-insulin microspheres administered subcutaneously as a single injection produced <1% release of insulin in the first day but then lowered the serum glucose levels of diabetic rats to values <200 mg/dL for approximately nine days. When the doses were given at seven-day intervals, steady-state drug levels were achieved after only two doses. PEG-insulin PLGA microparticles show promise as a once-weekly dosed, sustained-release insulin formulation.

Shenoy et al. [243] developed an injectable, depot-forming drug delivery system for insulin based on microparticles technology to maintain constant plasma drug concentrations over a prolonged period of time for the effective control of blood sugar levels. Formulations were optimized with two well-characterized biodegradable polymers, namely PLGA and poly- ϵ -caprolactone, and evaluated *in vitro* for

physicochemical characteristics, drug release in phosphate-buffered saline (pH 7.4), and evaluated in vivo in streptozotocin-induced hypoglycemic rats. With a large volume of internal aqueous phase during a w/o/w double-emulsion solvent evaporation process and high molecular weight of the polymers used, they could not achieve high drug capture and precise control over subsequent release within the study period of 60 days. However, this investigation revealed that upon subcutaneous injection the biodegradable depot-forming polymeric microspheres controlled the drug release and plasma sugar levels more efficiently than plain insulin injection. Preliminary pharmacokinetic evaluation exhibited steady plasma insulin concentration during the study period. These formulations, with their reduced frequency of administration and better control over drug disposition, may provide an economic benefit to the user compared with products currently available for diabetes control.

Interferon α_{2a} (IFN α_{2a}) Interferon α_{2a} is indicated for the treatment of adults with chronic hepatitis C virus infection who have compensated liver disease and have not been previously treated with interferon α . To improve the stability and loading efficiency of protein drugs, a new microsphere delivery system comprises calcium alginate cores surrounded by PELA [poly-*D,L*-lactide-poly-(ethylene glycol)]. Recombinant IFN α_{2a} as a model drug was entrapped within calcium alginate cores surrounded by PELA by a w/o/w multiple-emulsion technique [244]. Core-coated microspheres stabilized the IFN in the PELA matrix. The core-coated microspheres indicated high encapsulation efficiency and biological retention as compared to conventional PLGA microspheres. The extent of burst release reduced to 14% in core-coated microspheres from 31% in conventional microspheres, indicating a new approach for water-soluble macromolecular drug delivery.

5.2.7 CONCLUSION

From this chapter, it has become apparent that a number microencapsulation methods are available today for the preparation of microspheres on an industrial scale. In fact, parenteral drug delivery systems based upon biodegradable microspheres are a true success story for the concept of drug delivery. However, the production of biodegradable microspheres containing a stable therapeutic peptide or protein still remains a major challenge in terms of technical obstacles. Ideally, peptides/proteins of therapeutic interest should be studied case by case, so as to bring to the fore processing steps and stress factors which damage them. Continued efforts to establish methods for stable protein, especially antigen, delivery from microspheres may hopefully pave the way for future microsphere-based vaccines. Areas of further research should focus on the performance of peptide/protein-loaded microspheres under in vitro and in vivo conditions. Interestingly, the addition of medium-chain triglycerides (MCT) modifies/shifts the triphasic release pattern of leuprolide acetate-loaded PLGA microspheres to a more continuous release in vitro [245]. Alternatively, BSA-loaded PLGA microspheres were coated with a thermo-sensitive gel, Pluronic F127 (PF127) [246]. The results demonstrated that PF127, which gelled at 37°C, inhibited the initial burst release of BSA from microspheres effectively. It is anticipated that more efforts will be invested in the future to develop

novel ways to reduce the initial burst release of entrapped peptide/protein and to attain a more continuous release. In addition, an *in vitro* release model mimicking the fate of biodegradable microspheres applied through the parenteral route would be highly desirable. Also, new strategies to stabilize proteins in microspheres during manufacturing, shelf life, or *in vivo* could be of general interest. Moreover, the use of analytical techniques such as FTIR or MALDI-TOF mass spectrometry certainly constitutes a step forward for protein analysis in more appropriate conditions.

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